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# Study of a Growth Factor for *Mycobacterium lepraemurium* I. Minimal Medium [1]

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It has been reported that *Mycobacterium lepraemurium* can be cultivated on 1% Ogawa yolk medium ([4,5,8]). Clarification of the reasons for this growth may hold some keys to the problem of cultivating *Mycobacterium leprae*. In Ogawa's ([8]) early report, it was noted that small inoculums of *M. lepraemurium* would not initiate growth on the 1% Ogawa yolk medium and even with heavy inoculums there were always found to be some cultures without growth, the bacilli disappearing even in the absence of contaminants. From these findings it would seem that the growth supporting factors of *M. lepraemurium* may be complicated. Cultivation from a small inoculum will be reported elsewhere ([6]). The present study concerns an investigation of a growth supporting factor in the presence of a heavy inoculum.

## MATERIALS AND METHODS

**Preparation method for modifications of 1% Ogawa yolk medium** ([7]). One percent Ogawa basal medium containing 15 μg/ml hemin, which was dissolved in trimethylamine water (80 mg/ml), was autoclaved and various additional substrates were mixed in this basal medium. Mixtures of one part of the basal medium and two parts of egg white or of yolk were divided into test tubes and sterilized as a slant medium for 40 minutes at 85°C the first time, and for 40 minutes at 80°C a second time. Special agar noble was mixed in the basal medium and autoclaved. After sterilization the agar medium was kept at 50°C to prevent coagulation and was mixed with one of the proteins listed below and divided into test tubes and sterilized as above. The oily fraction, which was difficult to mix with the water fraction, was suspended well by sonication. The pH of the medium was adjusted to pH 6.1-6.2 with calcium chloride and 1 N HCl. When the special agar noble was melted in the basal medium it was difficult to measure the pH at 50°C. Then the needed protein fraction was adjusted to pH 6.3 and mixed into the agar basal medium because the pH of the egg yolk was kept at 6.3 in making the 1% Ogawa yolk medium. When the residual powder after petroleum ether and acetone extraction and soy bean powder were used to make the slant medium, small bubbles in the medium mix-

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ture had to be removed in a vacuum dessicator to prevent a porous surface.

**Egg white minimal medium.** Mixture of 100 ml of the 1% Ogawa basal medium and 200 ml of egg white and 5 ml of 2% malachite green solution was adjusted to pH 6.1 with 1 N HCl, divided into test tubes and sterilized as above.

**Petroleum ether extraction from egg yolk.** Egg yolk was diluted with two times its volume of distilled water and lyophilized. Egg yolk powder was placed in a separation funnel which was stuffed with glass wool at its base and extracted repeatedly with petroleum ether for about ten days until the extracted petroleum ether became colorless. In the first extraction bubbles developing in the yolk powder were removed with a vacuum dessicator.

**DEAE-cellulose treatment of methanol extracted egg yolk fraction ([9]).** Egg yolk was mixed with water saturated butanol in a separation funnel in which the funnel hole was stuffed with glass wool. Butanol extraction was repeated and the extracted butanol was collected until it became colorless. The butanol extract was evaporated with a rotary evaporator, adding a small amount of water. The butanol extracted lipid fraction was dissolved in methanol and the methanol soluble fraction was collected. After activation of DEAE-cellulose with 0.5 N NaOH and 0.5 N HCl, methanol was added to the DEAE-cellulose to remove the water. Methanol soluble egg yolk fraction was applied on the methanol saturated DEAE-cellulose column. The passing through fraction was collected in an evaporating flask and after washing well with methanol, a phosphoryl isoprenoid alcohol fraction was eluted with 1% acetic acid containing 3% ammonium acetate ([2]).

**Extraction method for dolichol.** An unsaponified lipid fraction prepared from the liver of chickens according to the method of Burgos *et al* ([1]) was set on the almina column and eluted stepwise with petroleum ether containing 2% ethyl ether, 15% ethyl ether, and 30% ethyl ether. Vacuum dried, these crude fractions were used in the media.

**Evaluation of bacillary growth.** About 20 mg of *M. lepraemurium* grown on the 1% Ogawa yolk medium for two months were inoculated on the center of the medium. The growth was judged after two to four months as follows: (+++) more than approximately 100 mg; (++) about 50 mg; (+) about 30 mg; and (—) less than 20 mg.

**Experimental materials.** Hemin, biotin, $\beta$-carotin, tri-ethylene-diamine, cholesterol, egg white albumin and bovine serum albumin were purchased from Wako Pure Chemical Corporation. Farnesol, 2-aminoethyl-isothio-uranium-bromide-hydrobromide and retinol were products from the Sigma Corporation. Casamino acid and lyophilized yeast extract were from the Difco Corporation; lecithin was from the Nakarai Chemical Corporation; cysteamine was from the Tokyo Chemical Industry Corporation; soybean powder was "House Hon Dohfu" from the House Food Corporation; and DEAE-cellulose 52 was purchased from the Whatman Corporation.

## RESULTS

Many experiments directed at establishing a minimal medium were designed in order to study the growth factors for *M. lepraemurium*. After the egg yolks were well extracted with organic solvents, the residual fractions were used to make the slant media. As seen in Table 1, both residual fractions left after extraction with petroleum ether or acetone permitted the growth of *M. lepraemurium*. As both residual fractions from butanol or alcohol extraction were not coagulable, these residual fractions could not be used to make the slant media. Koseki and Anchi ([3]) reported that *M. lepraemurium* could grow on 1% Ogawa whole egg medium which was adjusted to pH 6.3 with $CaCl_2$ or HCl. When I tried this method, *M. lepraemurium* grew well on the 1% Ogawa whole egg medium at pH 6.3, as seen in Table 2. It was thought that *M. lepraemurium* might grow on every medium if the pH was adjusted to 6.1. However, *M. lepraemurium* did not grow on several media adjusted to this pH, as seen in Tables 2-4. Soybean powder, horse serum, milk and egg white were used for protein fraction instead of egg yolk protein and supplemented with yeast extract and biotin, but *M. lepraemurium* did not grow markedly on these media (Table 2). Milk or egg white medium gave relatively good results. Colonies grown on egg white medium for two months could be passaged to 1% Ogawa yolk medium. Egg white albumin or bovine serum albumin were used for the protein fraction

and casamino acid, tryptophan, yeast extract, cysteine, broth and biotin were used in combinations to supplement nutrition, as detailed in Tables 3 and 4, with the media adjusted to pH 6.1. These media did not support the growth of *M. lepraemurium* and the organisms could not be passaged to 1% Ogawa yolk medium after two months. From these results it was concluded that egg white medium adjusted to pH 6.1 was most suitable as a minimal nutrition medium for *M. lepraemurium*.

Growth factors for *M. lepraemurium* were investigated by testing their growth promoting action in the minimal medium supplemented with various substances from egg yolk. Since there is much lecithin and cholesterol contained in egg yolk, lecithin which was extracted from the yolk and synthesized cholesterol were added to the minimal medium. At the same time, petroleum ether extractable yolk fraction or retinol, which was chosen because it carries an isoprenoid alcohol on the side chain, were also added to the minimal medium. No such media supported the growth of *M. lepraemurium*, as shown in Table 5. However, relatively good results were seen with the retinol medium.

TABLE 1. *Cultivation of* Mycobacterium lepraemurium *on modified 1% Ogawa yolk media prepared with petroleum ether and acetone extracted residue of egg yolk.*

| Media | Culture days | Growth[a] +++ |
|---|---|---|
| Petroleum ether extracted residue | 60 | 22 |
| Petroleum ether extracted residue with added oil fraction from petroleum ether soluble lipid | 60 | 20 |
| Acetone dry fraction of egg yolk | 63 | 42 |
| Acetone dry fraction of egg yolk without hemin | 63 | 38 |

[a] Number of media tubes.

TABLE 2. *Cultivation of* Mycobacterium lepraemurium *on modified 1% Ogawa yolk media prepared with various proteins.*

| Media | Culture days | Growth[a] | | | | |
|---|---|---|---|---|---|---|
| | | +++ | ++ | + | ± | — |
| Whole egg medium, pH 6.3 with $CaCl_2 \cdot 2H_2O$ | 95 | 50 | | | | |
| Whole egg medium, pH 6.3 with 2 N HCl | 95 | 54 | | | 2 | |
| "House Hon Dohfu" solidified with special agar noble, pH 6.2 with 2 N HCl[b, c] | 152 | | | | | 16 |
| Milk solidified with special agar noble, pH 6.2 with 2 N HCl[b, c] | 152 | | 21 | | | |
| Horse serum added Difco yeast extract 6.6 mg/ml solidified with special agar noble, pH 6.2 with 2 N HCl[b] | 102 | | | 19 | | |
| Horse serum added biotin, 0.36 mg/ml, solidified with special agar noble, pH 6.2 with 2 N HCl[b] | 102 | | | 18 | | |
| Egg white minimal medium, pH 6.2 with $CaCl_2 \cdot 2H_2O$ and 2 N HCl | 102 | | 40 | | | |

[a] Number of media tubes.
[b] Solidified with special agar noble.
[c] Soybean powder.

When the growth promoting activity of retinol was tested on retinol containing glycerin Sauton agar and glycerin broth agar no growth was observed as noted in Table 6. The butanol extractable fraction of yolk containing isoprenoid alcohol compounds and the DEAE-cellulose adsorped fraction is a phosphate ester of isoprenoid alcohol ([9]). The butanol fraction of yolk and the eluted fraction from DEAE-cellulose with ammonium sulfate acetic acid was used in the minimal medium. As seen in Table 7, none of these substrates supported the growth of *M. lepraemurium*. Since the growth of *M. lepraemurium* is enhanced in low oxygen tension ([6]), the minimal medium with the added butanol fraction of yolk was used under nitrogen containing 5% $CO_2$ and 1% $O_2$ or 5% $CO_2$ and 0.1% $O_2$ or 5% $CO_2$ and 0.01% $O_2$. The results were negative as recorded in Table 8. Since the growth supporting substances of *M. lepraemurium* may lie in a lipoprotein fraction of egg yolk which might destroy a free radical produced by the respiration of *M. lepraemurium* under oxygen, several neutralizing agents of free radicals were added to the minimal medium. As seen in Table 9, all reagents were ineffective for the support

TABLE 3. *Cultivation of* Mycobacterium lepraemurium *on minimal and albumin media.*

| Media | Culture days | Growth[a] ± — |
|---|---|---|
| Egg white minimal medium supplemented with 1% Difco yeast extract | 82 | 380 |
| Egg white minimal medium with 2 ml of 2% malachite green in 50 ml basal medium | 89 | 315[b] |
| Egg white albumin, 10 gm/100 ml, adjusted to pH 6.3. Mixed with hot 1% Ogawa basal medium containing 15γ/ml hemin and 3% special agar noble | 90 | 22 |
| Bovine serum albumin, 10 gm/100 ml in place of above egg white | 90 | 22 |

[a] Number of media tubes.
[b] Some colonies grew on 1% Ogawa yolk medium on reinoculation after two months.

TABLE 4. *Cultivation of* Mycobacterium lepraemurium *on albumin agar media.*

| Media | Culture days | Growth[a] |
|---|---|---|
| 1) Egg white albumin 0.7 gm/ml, hemin 5γ/ml & 1% special agar noble, pH 6.1 | 90 | 5 |
| 1′) Bovine serum albumin 0.7 gm/ml as above | 90 | 5 |
| 2) Casamino acid 100 mg & tryptophan 10 mg added to medium 1 | 90 | 5 |
| 2′) The above substrates in medium 1′ | 90 | 5 |
| 3) Yeast extract 50 mg & cysteine 10 mg in medium 2 | 90 | 5 |
| 3′) The above substrates in medium 2′ | 90 | 5 |
| 4) Bouillon 100 mg in medium 1 | 90 | 5 |
| 4′) Bouillon 100 mg in medium 1′ | 90 | 5 |
| 5) Biotin 5 mg in medium 2 | 90 | 5 |
| 5′) Biotin 5 mg in medium 2′ | 90 | 5 |

[a] Number of media tubes.

of growth of *M. lepraemurium*. In the use of cysteamine, the inoculated organisms were dyed blue with malachite green added to the medium after five days of cultivation. They were presumed to be dead. As the retinol had shown a relatively good growth supporting effect, some isoprenoid alcohol derivatives, farnesol (which is an insect hormone) and a dolichol fraction of chicken liver (which is a saccharide carrier for the biosynthesis of glycoprotein in mammalian tissue), were used to test possible growth supporting activity of *M. lepraemurium* on the minimal medium. Total lack of positive results are shown in Table 10.

## DISCUSSION

Minimal nutrition medium was used for the investigation of growth factors of *M. lepraemurium*. Several proteins, with the exception of egg protein, were not suitable in promoting growth of *M. lepraemurium*. Since *M. lepraemurium* will grow on whole egg medium at pH 6.1, egg white slant was adjusted to pH 6.1 and was found to be most suitable as a minimal medium. Butanol extract of yolk, lecithin, cholesterol, neutralizing agents of free radicals, retinol, farnesol, dolichol fraction of chicken liver, and other substances were added to the minimal medium but there was no effect with respect to growth support for *M. lepraemurium*. Only egg yolk and residual protein fractions of egg yolk extracted with organic solvents supported growth of *M. lepraemurium*. Since a growth factor of *M. lepraemurium* was found in a high molecular substance such as lipoprotein, there are three theoretical probabilities for this action of the egg yolk lipoprotein fraction. First, it may serve as a nutrient. However, such a high molecular lipoprotein fraction cannot be incorporated into the mycobacterial cell as such. Therefore, a low molecular, active subtance should be pre-

TABLE 5. *Cultivation of* Mycobacterium lepraemurium *on minimal medium supplemented with lecithin, cholesterol and retinol.*

| Supplemented substrates in basal medium | Culture days | Growth[a] | | | |
|---|---|---|---|---|---|
| | | ++ | + | ± | — |
| Lecithin 3.3 mg/ml | 154 | | 9 | 9 | |
| Lecithin 6.6 mg/ml | 154 | | 9 | 9 | |
| Lecithin 13.2 mg/ml | 154 | | | 7 | 10 |
| Cholesterol 3.3 mg/ml | 154 | | | 6 | 10 |
| Cholesterol 6.6 mg/ml | 154 | | | 7 | 10 |
| Cholesterol 13.2 mg/ml | 154 | | 4 | 3 | 10 |
| Lecithin 3.3 mg/ml, cholesterol 3.3 mg/ml | 154 | | 3 | 4 | 10 |
| Lecithin 6.6 mg/ml, cholesterol 6.6 mg/ml | 154 | 9 | | 7 | 1 |
| Lecithin 13.2 mg/ml, cholesterol 13.2 mg/ml | 154 | | 10 | | 8 |
| Petroleum ether soluble fraction 13.2 mg/ml | 154 | | 10 | 9 | |
| Retinol 0.3 mg/ml | 154 | 7 | 10 | 1 | |

[a] Number of media tubes.

TABLE 6. *Cultivation of* Mycobacterium lepraemurium *on Sauton and bouillon agar media.*

| Agar media | Culture days | Growth[a] |
|---|---|---|
| 1.5% special agar noble, Sauton, pH 6.2 | 90 | 5 |
| Retinol, 10 mg/30 ml in above | 90 | 5 |
| 1.5% special agar noble bouillon, pH 6.2 | 90 | 5 |
| Retinol, 10 mg/30 ml, in above | 90 | 5 |

[a] Number of media tubes

pared from the high molecular lipoprotein fraction. Second, it may serve as a regulator substance on the physical property of the surface of the medium. Third, it may be a neutralizing agent of some inhibitor contained in egg white. In our next investigations, the protein fractions of egg yolk will be studied with respect to these three concepts.

## SUMMARY

A growth promoting factor is contained in the petroleum ether or acetone extracted residue of lyophilized dry egg yolk. Egg white, horse serum, soybean powder, bovine serum albumin, egg albumin and milk were used in *M. lepraemurium* culture at-

TABLE 7. *Cultivation of* Mycobacterium lepraemurium *on minimal medium supplemented with organic solvent soluble fraction of yolk.*

| Media | Culture days | Growth[a] | | |
|---|---|---|---|---|
| | | +++ | + | — |
| 1% Ogawa yolk medium | 60 | 10 | | |
| Whole egg 1% Ogawa medium, pH 6.2 | 60 | 15 | | |
| Minimal medium[b] | 60 | | | 14 |
| Minimal medium with 5 γ/ml hemin | 60 | | | 17 |
| Minimal medium with 0.5% Difco yeast extract | 60 | | | 14 |
| Minimal medium with 5 γ/ml hemin & 0.5% Difco yeast extract | 60 | | | 15 |
| Minimal medium with 0.5% Difco yeast extract & 15 gm/75 ml butanol extracted yolk fraction | 60 | | 11 | |
| Minimal medium with 0.5% Difco yeast extract & 5 gm/75 ml residue from methanol extraction of butanol fraction of yolk | 60 | | 12 | |
| Minimal medium with 0.5% Difco yeast extract & 15 gm/75 ml DEAE-cellulose passed fraction of methanol fraction of yolk | 60 | | | 15 |
| Minimal medium with 0.5% Difco yeast extract & eluted fraction DEAE-cellulose | 60 | | | 11 |

[a] Number of media tubes.
[b] Minimal medium: egg white and 1% Ogawa basal medium, pH 6.1.

TABLE 8. *Cultivation of* Mycobacterium lepraemurium *on minimal medium, with butanol extract of yolk, under low oxygen tension.*

| Media | Growth/numbers of tubes after 110 days | | | |
|---|---|---|---|---|
| | 5% $CO_2$ Air | 5% $CO_2$ 1% $O_2$ | 5% $CO_2$ 0.1% $O_2$ | 5% $CO_2$ 0.01% $O_2$ |
| 1% Ogawa yolk medium & hemin | +++5 | +++2 | +++3 | +++2 |
| Minimal medium & butanol extract of yolk[a] | —11 | —8 | ±6 | —8 |
| Minimal medium & DEAE passed fraction | —10 | —8 | —5 | —8 |
| Minimal medium & DEAE eluted fraction | —3 | —3 | —3 | —3 |

[a] Butanol extract of yolk which was prepared from five egg yolks was suspended in 50 ml of 1% Ogawa basal medium and mixed with 100 ml of pH 6.4 egg white.

tempts as protein sources instead of yolk lipoprotein. None of these substances promoted the growth of *M. lepraemurium.*

One percent egg white medium was prepared from the mixture of one part 1% Ogawa basal medium to two parts egg white, adjusted to pH 6.1. This medium does not permit the growth of *M. lepraemurium* but permits bacillary survival for two months. This medium is most suitable as a minimal medium to investigate growth factors of *M. lepraemurium.* Utilizing the minimal medium, the following substances were tested for growth promoting activity: lecithin, cholesterol, petroleum ether extracted fraction of yolk, butanol extracted fraction of yolk, retinol, hemin, yeast extract, broth, farnesol and dolichol fraction of chicken liver. None of these supported growth of *M. lepraemurium.*

The following neutralizing agents of free radicals were tried in the minimal medium: triethylenediamine, β-carotin, potassium iodide, potassium bromide, 2-aminoethyl-isothio-uranium-bromide-hydrobromide and cysteamine. None of these supported growth of *M. lepraemurium.*

TABLE 9. *Cultivation of* Mycobacterium lepraemurium *on minimal medium supplemented with hemin and radical capturing agents.*

| Radical capturing agents | Concentration | Growth at 96 days[a] | | |
|---|---|---|---|---|
| | | + | ± | — |
| Tri-ethylene-diamine | 0.33 mg/ml | 13 | | |
| | 0.66 mg/ml | 13 | | |
| | 0.99 mg/ml | 13 | | |
| β-carotin | 0.33 mg/ml | 9 | | 10 |
| Potassium iodide | 0.33 mg/ml | | 7 | 2 |
| | 0.66 mg/ml | | 3 | 6 |
| | 0.99 mg/ml | | 9 | |
| Potassium bromide | 0.33 mg/ml | | 7 | 2 |
| | 0.66 mg/ml | | 12 | |
| | 0.99 mg/ml | | 4 | 5 |
| 2-aminoethyl-isothio-uranium-bromide-hydrobromide | 0.33 mg/ml | 13 | | |
| | 0.66 mg/ml | 13 | | |
| | 0.99 mg/ml | 8 | | 5 |
| Cysteamine | 0.33 mg/ml | | | 12 |
| | 0.66 mg/ml | | | 9 |
| | 0.99 mg/ml | | | 9 |

[a] Number of media tubes.

TABLE 10. *Cultivation of* Mycobacterium lepraemurium *on minimal medium supplemented with isoprenoid alcohol derivatives and hemin.*

| Isoprenoid alcohol derivatives | Concentration | Growth at 62 days[a] | | |
|---|---|---|---|---|
| | | + | ± | — |
| Farnesol | 0.42 mg/ml | | | 25 |
| Dolichol fraction eluted with 30% ether | 0.66 mg/ml | | | 24 |
| Dolichol fraction eluted with 15% ether | 0.66 mg/ml | 12 | 3 | 9 |
| Dolichol fraction eluted with 2% ether | 0.66 mg/ml | 10 | 6 | 9 |

[a] Number of media tubes.

## RESUMEN

El residuo extraído de la yema de huevo liofilizada con éter de petróleo o acetona contiene un factor promotor del crecimiento. Se hicieron intentos de crecer al *M. lepraemurium* utilizando clara del huevo, suero de caballo, polvo de frijol soya, albúmina sérica bovina, albúmina de huevo y leche, como fuentes de proteína en substitución de la lipoproteína de la yema. Ninguna de estas substancias promovió el crecimiento del *M. lepraemurium.*

Se preparó un medio con 1% de clara de huevo a partir de la mezcla de una parte de medio basal de Ogawa al 1% y 2 partes de clara de huevo, ajustada al pH 6.1. Este medio no permite el crecimiento del *M. lepraemurium* pero permite la supervivencia bacilar durante 2 meses. Este medio es más apropiado como un medio mínimo para investigar factores de crecimiento para el *M. lepraemurium.* Utilizando el medio mínimo se probo la actividad promotora de crecimiento de las siguientes substancias: lecitina, colesterol, la fracción estraída de la yema con éter de petróleo, la fracción extraída de la yema con butanol, retinol, hemina, extracto de levadura, caldo y las fracciones extraídas del hígado de pollo con farnesol y dolicol. Ninguna de estas substancias promovió el crecimiento del *M. lepraemurium.*

También se probaron los siguientes neutralizantes de radicales libres: trietiléndiamina, $\beta$ -carotina, yoduro de potasio, el bromuro-hidrobromuro del 2-aminoetil-isotio-uranio, y cisteamina. Ninguno de estos promovió el crecimiento del *M. lepraemurium.*

## RÉSUMÉ

Il existe un facteur de simulation de la croissance dans l'ether de pétrole, de même que dans des résidus extraits par l'acétone de jaune d'oeuf séché et lyophilisé. Dans des essais visant à cultiver *M. lepraemurium,* on a tenté de remplacer les sources de protéine généralement fournies par la lipoprotéine du jaune d'oeuf, par du blanc d'oeuf, du sérum de cheval, de la poudre de soya, de l'albumine sérique de boeuf, de l'albumine d'oeuf, et du lait. Aucune de ces substances n'a stimulé la croissance de *M. lepraemurium.*

On a préparé un milieu à base de blanc d'oeuf à 1%, en mélangeant une part de milieu de base de Ogawa à 1% à deux parts de blanc d'oeuf, le tout étant ajusté au pH 6,1. Ce milieu ne permet pas d'obtenir une croissance de *M. lepraemurium,* mais par contre on assiste à une survie des bacilles pendant deux mois. Ce milieu convient fort bien comme milieu minimal pour explorer les facteurs de croissance de *M. lepraemurium.* En utilisant ce milieu minimal, on a étudié les activités de simulation de la croissance d'un certain nombre de substances: lécithine, cholestérol, éther de pétrole extrait d'une fraction de jaune d'oeuf, butanol extrait d'une fraction de jaune d'oeuf, rétinol, hémine, extrait de levure, bouillon, et fraction farnesol et dolichol du foie de poulet. Aucune de ces substances n'a entraîné une croissance de *M. lepraemurium.*

On a de même essayé les agents de neutralisation des radicaux libres suivants, dans le milieu minimal: triethylènediamine, $\beta$-carotène, iodure de potassium, bromure de potassium, 2-aminoethyl-isothiouranium-bromure-hydrobromure et cysteamine. Aucune de ces substances n'a stimulé la croissance de *M. lepraemurium.*


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